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GRK2-dependent S1PR1 desensitization is required for lymphocytes to overcome their attraction to blood

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Abstract

Lymphocytes egress from lymphoid organs in response to sphingosine-1-phosphate (S1P); minutes later they migrate from blood into tissue against the S1P gradient. The mechanisms facilitating cell movement against the gradient have not been defined. Here we show that G-protein coupled receptor kinase-2 (GRK2) functions in down-regulation of S1P receptor-1 (S1PR1) on blood-exposed lymphocytes. T- and B-cell movement from blood into lymph nodes is reduced in the absence of GRK2 but is restored in S1P-deficient mice. In the spleen, B-cell movement between the blood-rich marginal zone and follicles is disrupted by GRK2-deficiency and by mutation of an S1PR1 desensitization motif. Moreover, delivery of systemic antigen into follicles is impaired. Thus, GRK2-dependent S1PR1 desensitization allows lymphocytes to escape circulatory fluids and migrate into lymphoid tissues.

Blood and lymph contain high nM amounts of S1P and lymphocyte egress from lymphoid tissues is dependent on S1P triggering of S1PR1 on the lymphocyte (1). Within minutes of arriving in blood, lymphocytes are able to leave again (2). In lymph nodes (LNs), this occurs via high endothelial venules (HEVs) (3, 4). Localized chemokine-mediated Gi-signals activate integrins and are important for cell adhesion and subsequent transendothelial migration (5, 6). Yet S1PR1 is also a Gi-coupled receptor and its ligand is uniformly abundant in blood. How does a cell avoid being distracted by S1PR1 engagement and achieve the temporally restricted and correctly orientated Gi-signaling needed for LN entry. S1PR1 is down-modulated by ligand exposure in lymph and blood, but the mechanism for and importance of this down-modulation has not been determined (7). Here we tested the hypothesis that S1PR1 desensitization is required for T and B cells to overcome their attraction for blood and enter lymphoid tissue.

GRK2 is essential for mouse development but the key *in vivo* targets of this kinase are unknown (8–10). GRK2 is one of several GRKs expressed in T and B cells (11, 12) and *in vitro* studies indicate that it can phosphorylate S1PR1 (13, 14). We asked whether GRK2

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Supporting Online Material

Materials and Methods

Figs. S1 to S6

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had a non-redundant role in S1PR1 desensitization within T cells. Thymocyte development in GRK2^{f/-} CD4-cre mice appeared normal and T cell numbers in the spleen were in the wild-type (WT) range whereas numbers in LNs were reduced (fig. S1A). Strikingly, GRK2-deficient blood T cells had high surface S1PR1 compared to undetectable amounts on control blood T cells (Fig. 1A). S1PR1 on T cells from LNs was only slightly higher than control levels (Fig. 1A), consistent with low extracellular S1P concentrations in lymphoid organs (16). In contrast, surface expression of CCR7 and CXCR4 on T cells from LNs, sites of chemokine exposure, were similar between GRK2-deficient and WT cells (Fig. 1A). *In vitro*, GRK2-deficient T cells were resistant to S1P-mediated S1PR1 down-modulation even at μ M amounts, in contrast to the low nM sensitivity of WT cells (fig. S1B). GRK2-deficient T cells showed elevated chemotactic responses to S1P (Fig. 1B and fig. S1C) whereas response to CXCL12 was similar to controls and the response to CCL21 was reduced (Fig. 1B). This may indicate that transwell migration assays to S1P are usually limited in magnitude due to receptor desensitization occurring in many cells before they reach the lower chamber.

In 20 min and 20 hour co-transfer experiments, GRK2-deficient T cells showed a defect in LN entry while being represented similarly to control T cells in blood and spleen (Fig. 1C). Transfer into mice lacking circulatory S1P (15) overcame the entry defect (Fig. 1D) establishing that it was a consequence of S1P receptor signaling and was not due to a role of GRK2 in CCR7 signaling or in other pathways. Deficient and control T cells in the blood of recipient mice had comparable integrin levels (fig. S1D). To test for evidence of constitutive S1PR1 signaling by GRK2-deficient T cells in blood, we examined phosphorylation of Ezrin/Radixin/Moesin (ERM) proteins since Gi-signaling is sufficient to decrease pERM levels (16). A small but significant reduction in pERM was detected in GRK2-deficient blood T cells (fig. S1E). Reduced pERM was not observed in GRK2-deficient LN T cells (fig. S1E) or in blood cells after transfer into S1P-deficient recipients (fig. S1E). Despite the reduced LN entry efficiency, GRK2^{f/-} CD4-cre mice showed reduced rather than increased T cell numbers in blood, indicating that GRK2 is needed for T cell homeostasis (fig. S1A), perhaps reflecting a need to access LN-restricted trophic signals (17) or other undefined functions of GRK2 in T cells (10). Video-rate microscopy of LN HEVs revealed a 3–4 fold reduction in GRK2-deficient T cells undergoing the rolling-to-sticking transition compared to controls (Fig. 1E). Rolling velocity was also reduced (Fig. 1E) perhaps reflecting alterations in membrane surface properties due to constitutive S1P signaling (Fig. S1E) or the slightly elevated CD62L expression (fig. S1D).

Flow cytometric analysis of GRK2^{f/-} mice carrying a B cell-specific (Mb1-) cre demonstrated that B cells had a similar GRK2-dependence as T cells for ligand-mediated S1PR1 down-modulation in blood (Fig. 2A). The specificity of the antibody was confirmed by the lack of staining on S1PR1-deficient B cells (fig. S2A). Again the effect of GRK2 deficiency on surface GPCR expression showed selectivity for S1PR1 because expression of CXCR4, CXCR5 and CCR7 was similar to controls (Fig. 2A). GRK2-deficient B cells showed augmented chemotactic responses to S1P whereas responses to chemokines were in the normal range (Fig. 2B and fig. S2B). GRK2^{f/-} Mb1-cre mice had elevated splenic and blood B cell numbers but reduced numbers in LNs (fig. S2C). Unexpectedly, in short term (30 min) transfer experiments GRK2-deficient B cells accumulated in recipient LNs similarly to controls (Fig. 2C); however, after 20 hours fewer GRK2-deficient than control B cells had accumulated (Fig. 2C). When the B cells were resident for 20 hours in S1P-deficient hosts (15), GRK2-deficient and control cells entered LNs in similar numbers (Fig. 2D). The GRK2-deficient cells expressed similar amounts of integrins and L-selectin as control cells (fig. S2D). We then asked whether an early entry defect in GRK2-deficient B cells might be obscured by the attachment of large numbers of transferred B cells to HEVs. To measure the fraction of B cells that had entered the tissue versus remained within the

HEVs we treated mice for a few minutes with a phycoerythrin (PE)-conjugated antibody, a procedure that allows selective labeling of blood-exposed cells (fig. S2E) (18). This analysis revealed that a greater fraction of GRK2-deficient than WT LN B cells remained blood exposed 30 min after transfer (Fig. 2, E and F). This effect was S1P-dependent as it was lost in mice lacking circulatory S1P (Fig. 2F). A similar experiment with GRK2-deficient T cells did not reveal enrichment for blood-exposed cells (fig. S2F). Quantitative real-time measurement of HEV attachment is more difficult for B cells than T cells due to a ~10-fold lower rate of LN entry (19, 20). Although we have not excluded a possible effect of GRK2-deficiency on B cell rolling-to-sticking transitions, it appears that post-adhesion transmigration may be more dependent on S1PR1-desensitization in B cells than in T cells.

The spleen is unusual in having an open circulation, with blood being released in an area that surrounds the follicles, called the marginal zone (MZ) (21). Specialized B cells reside there, termed MZ B cells and they help deliver antigens into the follicles to promote humoral immunity (21–23). MZ B cell positioning in the MZ requires S1PR1 in the B cell to overcome attraction to follicular CXCL13 (24). We next asked whether MZ B cell movement from MZ into follicle requires GRK2-dependent S1PR1 desensitization.

MZ B cells from Mb1-cre GRK2-deficient mice had elevated surface S1PR1 whereas CXCR4, CXCR5 (Fig. 3A) and other markers were expressed normally (fig. S3A). In transwell migration assays, GRK2-deficient MZ B cells showed increased migration to S1P (Fig. 3B). CCL21 responses were reduced, like in T cells, suggesting GRK2 may augment CCR7 function in some cell types (fig. S2B). MZ B cells also express S1PR3 (23, 24). GRK2 deficiency augmented S1PR1 function, however, because the MZ B cells showed elevated responses to SEW2871 (Fig. 3B) an S1PR1-selective agonist (25).

To quantitate MZ B cell distribution we labeled blood-exposed B cells by injection of CD19-PE antibody. After 5 min, ~55% of WT MZ B cells were labeled (Fig. 3C); the remaining cells were protected from rapid antibody exposure due to their follicular localization (23). By contrast, ~80% of the GRK2-deficient MZ B cells became antibody labeled, indicating that a significantly greater fraction of these cells were situated in the MZ (Fig. 3C). An intermediate effect was observed in GRK2^{+/-} B cells (Fig. 3C). In S1PR1/GRK2 compound heterozygous mice, MZ B cell distribution returned to a state similar to WT (Fig. 3D). Accumulation of MZ B cells in the MZ was not due to indirect effects on other cell types as there was a similar increase in antibody labeling of GRK2-deficient cells in mixed WT:GRK2^{f/-} Mb1-cre BM chimeras (Fig. 3C). FTY720 treatment caused rapid down-modulation of S1PR1 and relocalization of wild-type MZ B cells to the follicle (fig. S3B) (24). These events were delayed in GRK2-deficient MZ B cells (fig. S3B), consistent with a role for GRK2 in FTY720-mediated S1PR1 down-modulation (26), and suggesting that S1PR1 function is needed in GRK2-deficient cells for retention in the MZ. In sections, GRK2-deficient MZ B cells were restricted to the MZ whereas control cells were distributed in both MZ and follicle (Fig. 3E).

Previous studies have identified several regions in the S1PR1 C-terminus that contribute to desensitization (13, 26–28). We established a role for the last 12 amino acids in supporting desensitization in a B cell line and mutating the TSS motif in this region to AAA (fig. S4A) led to a loss in sensitivity for S1P-mediated receptor down-modulation (fig. S4B). Mice carrying the TSS to AAA mutation in the endogenous S1PR1 locus (S1PR1^{TSS}; fig. S4C) had normal follicular and MZ B cells numbers (fig. S4D). By flow cytometry, MZ B cells from S1PR1^{TSS} mice showed higher S1PR1 expression than control cells (Fig. 4A). *In vitro*, S1PR1^{TSS} MZ B cells were resistant to S1P-mediated S1PR1 internalization (fig. S4E). In chemotaxis assays, MZ B cells showed augmented responses to S1P while retaining normal responses to CXCL12 and CXCL13 (Fig. 4B). *In vivo* CD19-PE labeling revealed an

accumulation of MZ B cells in the MZ (Fig. 4C). In sections, MZ B cells in S1PR1^{TSS} mice were restricted to the MZ (Fig. 4D). Transcriptional down-modulation of S1PR1 by LPS treatment (24) caused a similar follicular repositioning of WT and S1PR1^{TSS} MZ B cells (fig. S4F). These observations establish a requirement for S1PR1 desensitization in MZ B cell movement between MZ and follicle. Recirculating S1PR1^{TSS} T and B cells were not as desensitization resistant as GRK2-deficient T and B cells, as they showed almost complete S1PR1 down-modulation in blood (fig. S4G) and entered LNs with normal efficiency (fig. S4H), consistent with the presence of additional desensitization motifs in S1PR1 (13, 26–28). The different sensitivities of MZ and recirculating lymphocytes to the TSS mutation may reflect partially distinct modes of desensitization or be a consequence of lower S1P concentrations in MZ than blood.

In vivo labeling studies suggested that many MZ B cells migrate back and forth between MZ and follicle in an hour (23). Incubation of MZ B cells in blood concentrations of S1P led to partial downregulation of S1PR1 within 10 min and complete modulation by 30 min (Fig. 4E). Reciprocally, when MZ B cells were removed from exogenous S1P there was an increase in surface S1PR1 expression within 10 min, reaching maximum levels by ~30 min (Fig. 4E). *In vivo*, average S1PR1 surface levels were slightly lower on MZ B cells in the MZ compared to MZ B cells in the follicle, whereas CXCR5 levels were the same (Fig. 4F). These observations suggest MZ B cell movement between MZ and follicle due to S1PR1 desensitization in the S1P high MZ and resensitization in the S1P low follicle could occur in periods under an hour.

MZ B cells mediate delivery of immune complexes (ICs) to splenic FDCs (21–23). When control mice were treated to generate systemic PE-containing ICs, complexes could be visualized on MZ B cells within 1 hour (fig. S5) and by 18 hours they had been deposited on FDCs (Fig. 4G). In GRK2^{f/f} Mb1-cre mice, although similar amounts of PE-ICs were initially found on MZ B cells (fig. S5A), deposition on FDCs was reduced (Fig. 4G). A similar defect in IC deposition on splenic FDCs was observed in S1PR1^{TSS} mice (Fig. 4G). By contrast, B cell GRK2-deficient and S1PR1^{TSS} mice showed a similar ability to their matched controls to deposit subcutaneously forming ICs on draining LN FDCs (fig. S5C), a process that does not involve MZ B cells (29).

We identify a central role for GRK2 in ligand-mediated desensitization of lymphocyte S1PR1 (fig. S6). *In vitro* studies indicate that Gi-signals need to be received in a polarized fashion for T cells to undergo shear resistant adhesion (5, 6). By demonstrating that non-polarized S1PR1-mediated Gi-signaling disrupts the rolling-to-sticking transition in T cells, our data provide *in vivo* support for this model. Non-polarized S1PR1 signaling in GRK2-deficient cells also likely distracts cells from undergoing the polarized migration needed to cross the endothelium. Our results are also consistent with a model where cycles of S1PR1 de- and re-sensitization form the basis of a migratory oscillator that supports movement of MZ B cells between MZ and follicle (fig. S6). A general implication of these findings is that GRK2 antagonists may suppress lymphocyte migration from blood into tissue and thus could have therapeutic potential as immunosuppressants.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig 1.

GRK2-deficient T cells are resistant to S1PR1 desensitization and have a reduced ability to enter LNs. (A) Flow cytometric analysis of blood and peripheral LN (pLN) CD4 T cells from WT and GRK2^{f/f}-CD4Cre (KO) mice for S1PR1, CCR7 and CXCR4. Shaded histogram shows background (bg) staining of negative control. (B) Transwell migration of WT and KO CD4 T cells to S1P, CXCL12 or CCL21. Three replicates were run for each group. Bars indicate mean ($\pm$ SEM). (C) Short term in vivo homing efficiency of GRK2 KO T cells relative to co-transferred WT T cells. Transferred B cells are tracked as an additional control. bld, blood; spln, spleen; mLN, mesenteric LN. Data are from 3 experiments (n=8 mice). Bars indicate mean ($\pm$ SEM). (D) Short term in vivo homing efficiency of KO T cells relative to co-transferred WT T cells in WT and S1P-deficient recipients. Data are from 3 experiments (n=8 mice). Bars indicate mean ($\pm$ SEM). (E) Rolling-to-sticking transition frequency and rolling velocity (Vroll) of transferred WT and KO T cells visualized by intravital microscopy in pLN HEVs. Summary of 3 experiments (n=3 mice). Bars indicate mean ($\pm$ SEM). * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, ns, not significant ($p > 0.05$) by unpaired Student's t-test.

Fig. 2. GRK2-deficient follicular B cells are resistant to S1PR1 desensitization and have a reduced ability to enter LNs. (A) Flow cytometric analysis of blood and peripheral LN follicular B cells from WT and GRK2^{f/-} Mb1-cre mice (KO) for S1PR1, CXCR5, CXCR4 and CCR7. (B) Transwell migration of WT and KO B cells to S1P, CXCL12 and CXCL13. Three replicates were run for each group. Bars indicate mean ($\pm$ SEM). (C) Short term homing efficiency of KO B cells relative to co-transferred WT B cells. Transferred T cells are tracked as an additional control. Summary of 3 experiments (n=8 mice). Bars indicate mean ($\pm$ SEM). (D) Short term homing of GRK2-deficient B cells relative to co-transferred WT B cells in WT and S1P-deficient recipients. Summary of 4 experiments (n=11 mice). Bars indicate mean ($\pm$ SEM). (E) Flow cytometric detection of 30 min transferred B220+ B cells and CD3+ T cells labeled by 2 min in vivo anti-CD19-PE and anti-CD4/8-PE treatment. (F) Short term homing efficiency of KO B cells relative to cotransferred WT B cells in WT (n=10) and S1P-deficient (n=6) recipients, showing cells that were protected from anti-CD19/4/8-PE exposure (PE-) or blood exposed (PE+), identified as in F. Summary of 3 experiments. Bars indicate mean ($\pm$ SEM).

Fig. 3. MZ B cell migration into follicles requires GRK2-dependent S1PR1 desensitization. (A) Flow cytometric analysis of splenic MZ B cells from WT and GRK2^{f/f} Mb1-cre (KO) mice for S1PR1, CXCR5 and CXCR4. (B) Transwell migration of WT and KO MZ B cells to S1P, CXCL12, CXCL13 and S1PR1 agonist SEW2871. Three replicates were run for each group. Bars indicate mean ($\pm$ SEM). (C) Flow cytometric detection of 5 min in vivo anti-CD19-PE labeled MZ B cells from WT, GRK2^{f/f} Mb1-cre (KO) or GRK2^{+/-} (Het) mice shown as a histogram overlay (left panel) and a summary of data from 4 experiments (WT n=28, KO n=15, Het n=13 mice) (center panel). Right panel shows a summary of data from 3 experiments for WT and GRK2^{f/f} Mb1-cre (KO) cells present in mixed BM chimeras (n=8). Bars indicate mean ($\pm$ SEM). (D) Flow cytometric detection of 5 min in vivo anti-CD19-PE labeled MZ B cells from WT, GRK2^{+/-} (GRK2 het), S1PR1^{+/-} (S1PR1 het) and GRK2^{+/-} S1PR1^{+/-} (GRK2/S1PR1 het/het) mice shown as a histogram overlay (left panel) and a summary of data from 5 mice (right panel). Bars indicate mean ($\pm$ SEM). (E) Immunohistochemical localization of CD1d^{hi} MZ B cells (blue) with respect to the MAdCAM1⁺ marginal sinus (brown) in spleen sections from GRK2^{+/-} Mb1-cre (WT) and GRK2^{f/f} Mb1-cre (KO) mice. MZ, marginal zone. FO, follicle.

Fig. 4.

MZ B cell shuttling and immune complex delivery into follicles is disrupted by mutation of an S1PR1 desensitization motif. (A) Flow cytometric analysis of S1PR1 expression on MZ B cells from freshly isolated WT and S1PR1^{TSS} (TSS) mice. (B) Transwell migration of WT and TSS MZ B cells to S1P, CXCL12 and CXCL13. Three replicates were run for each group. Bars indicate mean ($\pm$ SEM). (C) Flow cytometric detection of 5 min *in vivo* anti-CD19-PE labeled MZ B cells from the indicated mice shown as a histogram overlay (left panel) and a summary of data from 9 mice. (D) Immunohistochemical localization of CD1d^{hi} MZ B cells (blue) with respect to the MAdCAM1⁺ marginal sinus (brown) in spleen sections from WT and TSS mice. (E) Surface S1PR1 internalization and recovery on MZ B cells exposed to 1 μ M S1P (internalization) or removed from S1P exposure (recovery) for 10, 30 or 60 min. Shaded histogram shows background (bg) staining of negative control. (F) Flow cytometric analysis of S1PR1 and CXCR5 expression on 5 min *in vivo* CD45.2 antibody labeled ('MZ') and unlabeled ('FO') MZ B cells. Shaded histogram shows background (bg) staining of negative control. Right panel shows ratio of S1PR1 or CXCR5 mean fluorescence intensities (MFI) of MZ B cells in the two compartments from 3 independent experiments. (G) Immunofluorescence microscopy of spleen sections taken from the indicated mice 18 h after treatment to form systemic PE-ICs (red), stained to detect marginal sinus-associated macrophages (CD169⁺, green) and FDCs (complement receptor 1 (CR1)⁺, blue). All data are representative of at least 3 mice of each type.